

FORM PTO-1390 (Modified)
(REV 10-95)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES

46793

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

09/269321

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/US97/17143

24 September 1997

24 September 1996

TITLE OF INVENTION

METHOD OF TARGETING MALIGNANT CELLS USING AN E2F RESPONSIVE PROMOTER

APPLICANT(S) FOR DO/EO/US

Howard A. FINE and William KAELEN, Jr.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☐ Other items or information:

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR		INTERNATIONAL APPLICATION NO. PCT/US97/17143		ATTORNEY'S DOCKET NUMBER 46793	
<div style="display: flex; justify-content: space-between;"><div><p>20. The following fees are submitted:</p><p>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :</p><div style="display: flex; justify-content: space-between;"><div><input checked="" type="checkbox"/> Search Report has been prepared by the EPO or JPO</div><div>\$840.00</div></div><div style="display: flex; justify-content: space-between;"><div><input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482)</div><div>\$670.00</div></div><div style="display: flex; justify-content: space-between;"><div><input type="checkbox"/> No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))</div><div>\$760.00</div></div><div style="display: flex; justify-content: space-between;"><div><input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO</div><div>\$970.00</div></div><div style="display: flex; justify-content: space-between;"><div><input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)</div><div>\$96.00</div></div></div><div style="text-align: center; margin-top: 10px;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div></div> <div style="border: 1px solid black; padding: 5px; width: fit-content;">CALCULATIONS PTO USE ONLY</div>					

Surcharge of **\$130.00** for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)). ☐ 20 ☒ 30

\$840.00

\$130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	15 - 20 =	0	x \$18.00	\$0.00	
Independent claims	1 - 3 =	0	x \$78.00	\$0.00	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$970.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). <input type="checkbox"/>				\$0.00	
SUBTOTAL =				\$970.00	
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30 +				\$0.00	
TOTAL NATIONAL FEE =				\$970.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL FEES ENCLOSED =				\$970.00	
				Amount to be: refunded	\$
				charged	\$

☒ A check in the amount of **\$970.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **50-0850** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Ronald I. Eisenstein
PEABODY & BROWN
101 Federal Street
Boston, MA 02110
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SIGNATURE

Ronald I. Eisenstein
NAME

30,628
REGISTRATION NUMBER

24 March 1999
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Howard A. FINE and William KAE LIN, Jr.
Serial No.: Not Yet Assigned
Filed on: Herewith
Title: METHOD OF TARGETING MALIGNANT CELLS USING AN E2F
RESPONSE RESPONSE

Sir:

STATEMENT CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(b)--NONPROFIT ORGANIZATION)

I hereby state that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Nonprofit Organization Dana-Farber Cancer Institute, Inc.
Address of Nonprofit Organization 44 Binney Street, Boston, Massachusetts 02115

TYPE OF NONPROFIT ORGANIZATION

- ☐ University or Other Institution of Higher Education
☒ Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501 (c)(3))
☐ Nonprofit Scientific or Educational Under Statute of State of the United States of America
(Name of State _____)
(Citation of Statute _____)
☐ Would Qualify as Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)), if Located in the United States of America
☐ Would Qualify as Nonprofit Scientific or Educational Under Statute of State of the United States of America if Located in the United States of America
(Name of State _____)
(Citation of Statute _____)

I hereby state that the nonprofit organization identified above qualifies as a nonprofit organization, as defined in 37 CFR 1.9(e), for purposes of paying reduced fees to the United States Patent and Trademark Office under Sections 41(a) and (b) of Title 35, United States Code, with regard to the invention described in

- ☒ the specification filed herewith, with title as listed above.
☐ the application identified above.
☐ the patent identified above.

I hereby state that rights under contract or law have been conveyed to, and remain with, the nonprofit organization, with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. 1.9(c), if that person made the invention, or by any concern that would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e)

*NOTE: Separate statements are required from each named person, concern or organization having rights to the invention as to their status as small entities. (37 CFR 1.27).

Each such person, concern or organization having any rights in the invention is listed below:

- ☐ No such person, concern, or organization exists.
☒ Each such person, concern or organization is listed below.

FULL NAME _____
ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME _____
ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any charge in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

(check the following item, if desired)

NOTE: The following verification statement need not be made in accordance with the rules published on October 10, 1997, 62 Fed. Reg. 52131, effective December 1, 1997.

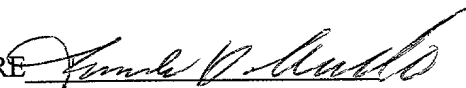
NOTE: "The presentation to the Office (whether by signing, filing, submitting, or later advocating) of any paper by a party, whether a practitioner or non-practitioner, constitutes a certification under § 10.18(b) of this chapter. Violations of § 10.18(b)(2) of this chapter by a party, whether a practitioner or non-practitioner, may result in the imposition of sanctions under § 10.18(c) of this chapter. Any practitioner violating § 10.18(b) may also be subject to disciplinary action. See §§ 10.18(d) and 10.23(c)(15)." 37 CFR 1.4(d)(2).

☒ I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing
Title in Organization
Address of Person Signing

Pamela D. Ariniello
Patent Counsel
Dana-Farber Cancer Institute, Inc.
44 Binney Street, Boston, MA 02115

SIGNATURE



Date

August 30, 1995

09120902..
510 Rec'd PCT/PTO 24 MAR 1999

Docket No. 46793

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Howard A. FINE and William KAELIN, Jr.
SERIAL NO.: Not Yet Assigned
[Express Mail Label No. EL310196101US]
FILED: Herewith
FOR: METHOD OF TARGETING MALIGNANT CELLS USING AN E2F
RESPONSE PROMOTER

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, DC 20231

SIR:

PRELIMINARY AMENDMENT

Please cancel claims 1-14.

IN THE CLAIMS

Claims 15 and 16, line 1, delete "14", and insert --25--.

Claim 17, line 1, delete "11", and insert --16--.

Claim 20, line 2, after "gene", insert --, a dominant negative mutant--.

Claim 24, line 1, delete "cytotoxic" and insert "cytotoxin"

Please add the following new claims:

- 25. A method of selectively expressing a gene in a malignant cell comprising:
- (a) determining whether a malignant cell expresses sufficient E2F to cause expression of a gene operably linked to an E2F responsive promoter;
 - (b) adding an effective amount of a nucleic acid cassette to the malignant cell that you determined expresses sufficient E2F, wherein said nucleic acid cassette comprises

an E2F responsive promoter operable linked to a gene of interest, wherein said gene is a positive potentiator or a negative potentiator; and

(c) waiting until the nucleic acid cassette transduces the malignant cell and expresses the gene.

26. The method of claim 25, wherein the E2F responsive promoter is selected from the group of promoters consisting of E2F1 promoter, dihydrofolate reductase promoter, DNA polymerase α promoter, c-myc promoter and β -myb promoter.--

REMARKS

Applicants have amended the method claims, rewritten as claims 25 and 26 to specify that the malignant cell targeted by the present method is one that will not repress an E2F responsive promoter. This is taught in the specification at pages 10-11. Claim 26 is supported by the specification at page 9. As such these amendments do not constitute new matter and their entry is respectfully requested.

Early and favorable action is requested.

Respectfully submitted,



Ronald I. Eisenstein (Reg. 30,628)
Peabody & Brown
101 Federal Street
Boston, MA 02110
(617) 345-6054

Date: March 24, 1999

510 Rec'd PCT/PTO 24 MAR 1999

METHOD OF TARGETING MALIGNANT CELLS USING AN
E2F RESPONSIVE PROMOTER.

BACKGROUND OF THE INVENTION

1. Field

The present invention is directed to a method of selectively targeting malignant cells. More specifically it involves the use of an E2F responsive promoter operably linked to a gene whose expression is desired in a tumor cell, but not non-malignant cells.

2. Background

One of the difficulties in treating individuals with malignant cells exhibiting uncontrolled growth, such as typically associated with tumors including solid tumors, leukemia, etc., is that antineoplastic agents are not highly selective. For example, many agents (e.g. radiation, chemotherapy) do not distinguish between malignant and non-malignant cells, but are directed to dividing versus non-dividing cells. Thus, such agents do not effect quiescent malignant cells while adversely effecting normal dividing cells.

There have been proposals to use gene therapy techniques in treating malignant cells. For example, targeting a cell with a positive potentiator such as a cytokine or costimulatory molecule to enhance an immune reaction to the tumor or a suicide gene or cytotoxin to kill the malignant cells. Many proposals have focused on using retroviruses that preferentially infect actively dividing cells. However, not all the cells in a tumor are actively dividing at any one point in time. And it is well

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known that one of the problems in cancer treatment is from the cells not killed in the initial treatment. Moreover, not all actively dividing cells are tumor cells.

Alternatively strategies that focus on using a vector that will infect both resting and actively dividing cells such as adenoviral vectors also have problems. While some degree of selectivity can be obtained by means of administration, the vector will still infect the normal tissue surrounding the tumor as well as the tumor. Consequently, the treatment will also cross-over to the normal tissue. For example, delivery of a cytotoxin or suicide gene will lead to some degree of host toxicity due to delivery of the gene to the surrounding normal cells. Thus, methods for increasing selectivity between malignant and non-malignant (normal) cells would be desirable.

The E2F family (E2F1-E2F5) are capable of activating transcription when bound to DNA. For example, E2F-1 is a ubiquitously expressed growth regulated, gene exhibiting peak transcriptional activity in S-phase [Tevosian, S.G., et al., Cell Growth and Diff. 7:43-52 (1996), Kaelin, W.G. et al., Cell 70: 351-364 (1992)] Transcription of the gene is cell cycle dependent as a result of E2F DNA-binding sites within its promoter [Neuman, E., et al, Mol. Cell Biol. 14:6607-6615 (1994)]. E2F activity is regulated, in part, by complex formation with cell cycle

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regulatory proteins such as cyclin A, cyclin K, cdk2, and members at the retinoblastoma protein (pRB) family (pRB, p107 and p130) [Weinberg, R.A., Cell 81:323-330 (1995); Adams P.D. and Kaelin, W.G., Seminars in Cancer Biology 6 : 99-108 (1995)] Members of the pRB family actively repress transcription when bound to DNA via E2F [Sellers, W.R., et al. Pro. Natl. Acad. Sci. USA 92: 11544-11548 (1995); Weintraub, S.J. et al. Nature 358: 259-261 (1992)]. Overproduction of E2F can override a pRB-induced growth arrest [Qin X.-Q, Mol.Biol. Cell. 15: 742-755 (1995); Zhu. L, et al. Genes Dev. 7: 1111-1125 (1993)] Many malignant cells for examle solid tumors such as malignant gliomas, have disrupted pRB function; either due to RB-1 gene mutations or due to mutations affecting upstream regulators of pRB such as cyclin D1 or p16/INK 4aIMTSI [Weinberg RA, Cell, supra, He J. et al, Cancer Research 54:5804-5807 (1994)]; Schmidt, EE, et al. Cancer Research 54:6321-6324 (1994). Although this might suggest that greater levels of E2F are expressed in malignant cells, E2F is still expressed in non-malignant cells.

SUMMARY OF INVENTION

We have now discovered a system that is substantially more tumor-specific than current antineoplastic agents such as radiation and

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chemotherapeutic agents. This system uses a "vector system" containing an E2F responsive promoter operably linked to a heterologous gene of interest, preferably encoding a negative or positive potentiator, more preferably a negative potentiator such as a suicide protein or a cytotoxic protein. This system results in high selectivity *in vivo* between malignant and non-malignant cells. Preferably, the malignant cell is from a solid tumor.

The E2F transcription factor (sometimes referred to as E2F protein or E2F) can regulate expression of numerous genes effecting cellular proliferation including proto-oncogenes and genes regulating cell cycle progression. For example, the retinoblastoma tumor suppressor gene product (Rb) interacts with E2F to form a complex where E2F expression is inhibited.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a schematic of the Adenoviral vectors. The backbone adenoviral sequences are derived from an E3-deleted adenovirus serotype 5, with a bacterial plasmid insert in the E1a region. Following recombination, E1a is replaced by the early CMV promotor (Ad.CMV- β gal) or from the upstream region of the E2F-1 gene (basepairs -218 to +51), a segment containing 4 intact E2F, 1 NF- κ B, and 4 Sp1 consensus sequences. The

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β -galactosidase gene was derived from a Lac Z-containing adenoviral shuttle vector, previously described [25].

Figures 2A and b show in vitro cell cycle vector induction. Ad.E2F1- β gal cell cycle selective gene expression is shown. Fig. 2A shows the percentage of cells in S-phase in serum refed (\blacktriangle) versus serum starved C6 (Δ) cells. Figure 2B shows the relative β -galactosidase expression in C6 cells: (\bullet) Ad.CMV β gal, serum fed; (\circ) Ad.CMV- β gal, serum starved; (\blacksquare) Ad.E2F1- β gal serum fed; (\square) Ad.E2F1- β gal serum starved.

Figures 3A-F Ad.E2F1- β gal mediated tumor selective transgene expression in vivo:

Fig. 3A shows Ad.CMV- β gal injected into normal brain; Fig 3B shows Ad.CMV- β gal injected into tumor-bearing brain; Fig. 3C shows Ad.E2F1- β gal injected into normal brain; Fig. 3D shows Ad.E2F1- β gal injected into tumor bearing brain; Fig. 3E shows Ad.AE2F1- β gal injected into normal brain; Fig. 3F shows Ad.AE2F1- β gal injected into tumor bearing brain.

Figures 4A-4F show Ad.E2F1- β gal transduction of normal regenerating liver

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whole mount liver sections stained for β -galactosidase; Figures 4C and 4D show thin sections of liver tissue stained for β -galactosidase activity and PCNA expression; Figures 4E and 4F show immunofluorescence of thin sections of the liver for expression of the adenoviral fiber protein. Figures 4A, C and E show transduction by Ad.CMV- β gal; Figures 4B, D and F show transduction by Ad.E2F1- β gal.

Figures 5A-D show in vitro and in vivo anti-tumor activity of Ad.E2F1-tk.

In Fig. 5A

C6 glioma cells were infected with viral vectors at an MOI of 50 for 1 h, then washed, and replated in tissue culture plates containing GCV at various concentrations. The cells were fixed 72 h later, stained with methylene blue, and absorbance at 600 nm (A_{600}) was determined on a microplate reader. (Δ) no virus; (\circ) Ad.CMV- β gal; (\bullet) Ad.CMV-tk; (\square) Ad.E2F1- β gal; (\blacksquare) Ad.E2F1-tk.

Figure 5B shows Kaplan-Meier survival curves of animals treated by stereotactic injection of viral vectors into 6 d old established intracerebral C6 gliomas, followed by twice a day treatment with intraperitoneal GCV (15 mg/kg) or saline. (\bullet) Ad.CMV-tk, GCV; (\circ) Ad.CMV-tk, saline; (\blacksquare) Ad.E2F1-tk, GCV; (\square) Ad.E2F1-tk, saline; (\blacktriangle) Ad.E2F1- β gal, GCV; (Δ) Ad.E2F1- β gal, saline. Figures 5C and 5D show

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Hematoxylin-eosin stained thin section of brain from animals intracerebrally injected with Ad.CMV-tk (Fig. 5C) or Ad.E2F1-tk (Fig. 5D) and treated with CCV (15 mg/kg) twice daily for 7 d.

DETAILED DESCRIPTION OF THE INVENTION

Vector systems containing a E2F responsive promoter operably linked to a gene of interest can be used to selectively express that gene in significantly higher levels in a malignant cell in contrast to a non-malignant cell. The gene of interest is a gene whose expression is desired in the malignant cell but not the non-malignant cell. Preferably, the gene would be express a cytotoxic or therapeutic protein.

We have found that similar levels of expression of a suicide gene such as the herpes thymidine kinase (tk) gene are obtained in malignant cells whether the gene is operably linked to a E2F responsive promoter or another promoter such as the cytomegalovirus early promoter (CMV). This observation is based upon cell death based upon subsequent treatment with ganciclovir (GVC). Cells expressing tk are sensitive to GVC and are killed (See Fig. 5A and B). Animals treated with tk and GCV live significantly longer than untreated animals. However, whereas extensive tissue damage was seen in normal tissues injected with tk under the control of a CMV promoter after GVC treatment, no obvious

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normal tissue toxicity was seen in animals injected with tk operably linked to a E2F responsive promoter, except from that local trauma resulting from the injection, which was indistinguishable from sham injected animals (See Fig. 5C). Figure 4 further shows that the expression of a gene operably linked to an E2F responsive promoter is selective for malignant versus normal tissues. This selectivity is all the more remarkable because E2F is normally expressed in cells in a cycle dependent manner.

Although not wishing to be bound by theory we believe that there is an excess of "free" E2F, as well as loss of pRb/E2F repressor complexes in such cells, which results in the selective expression of for example the cytotoxic or therapeutic gene in the malignant cell.

Preferably, the E2F responsive promoter (sometimes referred to as the E2F promoter) is a mammalian E2F promoter, still more preferably it is a human E2F (i.e. has at least one E2F binding site) promoter. The E2F responsive promoter does not have to be the full length wild type promoter, but it must respond to a factor we believe to be E2F as determined by expression in a malignant cell having disruption of pRb function of a heterologous gene (sometimes referred to as transgene) under the promoters control as opposed to a lack of expression in the presence of pRb/E2F complexes. As explained in Sellers, EW.R., et al,

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Proc. Natl. Acad. Sci USA 92:11544-11548 (1995). E2F responsive promoters typically share common features such as Sp1 and/or ATF sites in proximity to their E2F site(s), which are frequently located near the transcription start site, and lack of a recognizable TATA box. E2F responsive promoters include E2F promoters such as the E2F1 promoter, dihydrofolate reductase (DHFR) promoter, DNA polymerase α (DPA) promoter, c-myc promoter and the B-myb promoter. The E2F1 promoter contains four E2F sites that act as transcriptional repressor elements in serum-starved cells. Preferably, an E2F responsive promoter has at least two E2F sites. However, additional E2F sites are not necessary.

As used herein "responsive" means a sequence having at least 70% wild-type E2F promoter activity in expressing a heterologous gene. This activity can be determined by a number of assays using known techniques based upon the present disclosure. For example, using a marker gene such as *E. coli* β -galactosidase (β gal) in an expression vector. For example, one can use a retroviral vector (e.g. a murine moloney leukemia virus such as pMV7), an adenoviral vector, a herpes simplex viral vector, etc. to transduce a glioma cell line (e.g. the 9L gliosarcoma cell line) operably linked to the E2F promoter. As a control,

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a vector with a different promoter can be used such as a CMV promoter or even a reference standard can be used. The transduced cells can then be screened for expression of β gal by known techniques. See Figures 1-5.

In normal tissues E2F responsive promoters are typically repressed by pRB/E2F complexes. The ability of pRB to act as a growth suppresser is linked to this property [Sellers, W.R. et al. Proc. Natl. Acad. Sci. U.S.A. 92:11544-11548 (1995)]. We demonstrated that the selectivity of the E2F responsive promoter for tumor cells as opposed to normal cells can be removed by introducing mutations into an E2F responsive promoter to remove the E2F responsive promoter to remove the E2F binding sites, Δ E2F responsive. See Figure 3E which shows β -galactosidase expression in normal cells, which is in contrast to a relative lack of expression in such cells transduced by an isogenic vector except for an intact E2F consensus sequence (Figure 3C). Moreover, expression in a tumor cell such as a glioma was less with the Δ E2F promoter than the isogenic E2F promoter except for intact E2F consensus sequences. (Fig. 3F). Although not wishing to be bound by theory we believe this is due to induction from "free" E2F.

The target cells include any malignant cells. For example,

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cancers, or leukemia such as solid tumors, preferably breast, kidney, liver, brains and colon cancers or leukemias. More preferably, a brain tumor such as glioma, or a liver tumor. Still more preferably, a brain tumor such as glioma.

One can readily determine if a malignant cell is an appropriate target tissue for a molecule by introducing a E2F vector with a marker gene into that cell and determining if the marker is expressed. If the marker is not expressed, it indicates the E2F responsive promoter is being repressed.

One can insert a cassette containing just the E2F responsive promoter operably linked to the heterologous gene into a cell. The cassette is a nucleic acid segment containing the E2F promoter operably linked to a nucleic acid segment encoding the heterologous gene of interest. Preferably the cassette is inserted in a vector. The cassette is preferably DNA. The cassette can be introduced into the target cell by any method which will result in the uptake and expression of the E2F cassette by the target cells. These can include vectors, liposomes, naked DNA, gene gun, adjuvant-assisted DNA, catheters, etc. Vectors include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or

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RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses and HIV-based viruses. One preferred HIV-based viral vector comprises at least two vectors wherein the *gag* and *pol* genes are from an HIV genome and the *env* gene is from another virus. DNA viral vectors are preferred. These vectors include herpes virus vectors such as a herpes simplex I virus (HSV) vector [Geller, A.I. et al. *J. Neurochem* 64: 487 (1995); Lim, F. et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); Geller, A.I. et al., *Proc Natl. Acad. Sci. U.S.A.* 90: 7603 (1993); Geller, A.I., et al., *Proc Natl. Acad. Sci USA* 87: 1149 (1990)], adenovirus vectors [LeGal LaSalle et al., *Science* 259: 988 (1993); Davidson, et al., *Nat. Genet* 3: 219 (1993); Yang, et al., *J. Virol.* 69: 2004 (1995)] and adeno-associated virus vectors [Kaplitt, M.G., et al., *Nat. Genet.* 8:148 (1994)].

Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the E2F

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cassette into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include naked DNA, CaPO_4 precipitation, DEAE dextran, electroporation, protoplast fusion, lipofecton, cell microinjection, viral vectors, etc.

For example, one can use the vector to target any desired target cell such as a glioma. For example, stereotaxic injection can be used to direct the vectors (e.g. adenovirus, HSV) to a desired location in the brain (e.g. a glioma). Stereotaxic surgery is performed using standard neurosurgical procedures (Pellegrino and Cushman, (1971)). Additionally, the vehicle containing the cassettes (or even "naked" cassettes) can be delivered by intracerebroventricular ("icv") infusion using a minipump infusion system, such as a SynchroMed Infusion System. A method based on bulk flow, termed convection, has also proven effective at delivering large molecules to extended areas of the brain and may be useful in delivering the vector to the target cell (Bobo et al., *Proc. Natl. Acad. Sci. USA* 91:2076-2080 (1994) *Am. J. Physiol.* 266:

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292-305 (1994); Morrison et al.,). Other methods that can be used including catheters, intravenous, parenteral, intraperitoneal and subcutaneous injection, oral or other known routes of administration.

One would inject a sufficient amount of the vector to obtain a concentration in the tissue containing the E2F cassette of the encoded protein ranging between about 1 pg/ml to 20 µg/ml. More preferably between 0.1 µg/ml to 10 µg/ml. Still more preferably, between about 0.5 µg/ml to 10 µg/ml.

The E2F responsive promoter can be combined with a desired nucleic acid sequence encoding a heterologous gene such as one for a positive potentiator (such as a gene for a cytokine or a costimulating molecules, a gene for a missing or defective protein, (many cancers are believed to result in part from missing or defective proteins such as tumor suppressor genes e.g. retinoblastoma, p53, others from changes from a proto-oncogene to an oncogene such as with ras, etc.) or a sequence for a negative potentiator (such as a toxin, an anti-sense RNA, a suicide gene such as HSV thymidiac kinase (tk), a ribozyme, a dominant-negative mutant, an antibody such as an antibody with an intracellular localization signal etc.). Negative potentiators are preferred. Toxins and suicide genes are more preferred. For example, when the

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nucleic acid encodes a toxin, one preferably takes care to alter the toxin gene to minimize its potential to affect nontargeted cells. This can be done by standard techniques such as deleting those sequences encoding recognition domains. Toxins are well known and include diphtheria toxin and truncated versions thereof, pseudomonas exotoxin, and truncated versions thereof, Ricin/abrin, Blocked ricin/abrin, Ricin ToxinA-chain, ribosome inactivating protein, etc. All these proteins have different domains. For example, the gene encoding PEA has several domains: Domain I is responsible for cell recognition, Domain II for translocation of the toxin cross-membrane and Domain III for adenosine diphosphate (ADP)-ribosylation of elongation factor 2, which is the step actually responsible for cell death. [Gary, G.L., et al., Proc. Natl. Acad. Sci. USA 81:2645-2649 (1984); Allured, V.S., et al., Proc. Natl. Acad. Sci. USA 83:13220-1324 (1986); Siegall, C.B., et al., J. Biol. Chem. 264:14256-14261 1989)]. Accordingly, by alterations in Domain I or Domain II, that render those domains incapable of expression, for example, by a frameshift mutation, insertion of termination sequences, or deletions one can minimize the ability of the toxin to affect neighboring cells. Thereafter, the skilled artisan can use standard techniques to insure that the other domains, or portions of domains where expression is desired, are used.

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For example, as indicated above, with PEA only Domain III is absolutely required. However, partial sequences from other domains makes the toxin more effective. For example, one can prepare PEA mammalian expression vectors in which Domain III (mature PEA amino acid residues 405 to 613) only, is expressed and one which encodes Domain III and partial Domain IB, a sequence of amino acids 385 to 613 is expressed. These sequences should be operably linked to the E2F responsive promoter which will permit expression in the target cell. For example, the human E2F-1 promoter. The toxin proteins encoded by these gene fragments lack a recognition domain. They are non-toxic to surrounding cells and are only toxic when expressed inside a cell. These expression vectors can readily be tested to determine how well they express a product intracellularly by a simple *in vitro* assay. For example, the expression of those DNA sequences encoding PEA toxin fragments can be tested by transforming an E2F high expressing malignant cell with the cassette and observing the cytotoxicity of the cell. Suicide genes such as tk work by sensitizing the cell to a compound which the cell would otherwise not be affected by, e.g. ganciclovir. One preferred type of antibodies works by binding to a target intracellularly (e.g. an intrabody). For example, by targetting a gene product that is overexpressed in malignant cells such as a gene erbB-2.

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In some instances, even with immunotoxins, resistant mutants can develop. In such instances, one can readily insert a different toxin gene or different types of nucleic acid segments into the E2F nucleic acid cassette. Thus, the present system permits the production and use of a wide range of DNA and RNA segments, DNA segments are preferred.

In some preferred embodiments one would administer a cocktail of different nucleic acid cassettes where the heterologous nucleic acid segment that is delivered is changed to widen the spectrum of products delivered to the target cell.

Combination therapy is particularly preferred. For example, the use of both a toxin and a suicide gene. Alternatively, one can use a cytokine or costimulating molecule to enhance an alternative strategy such as immune targetting of tumors. Thereafter, one can administer a toxin to the remaining cells. This can also be done with other anti-neoplastic agents such as radiation, chemotherapy, etc.

The E2F cassette may be administered alone, or as part of a pharmaceutical composition, together with one or more acceptable carriers thereof and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

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The compositions include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form, e.g., tablets and sustained release capsules, and in liposomes, and may be prepared by any methods well known in the art of pharmacy.

Such methods include the step of bringing into association with the molecule to be administered ingredients with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid, or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion, or packed in liposomes and as a bolus, etc.

A tablet may be made by compression or molding, optionally with

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one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture tablets may be made molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored any may be formulated so as to provide slow or controlled release of the active ingredient therein.

Compositions suitable for topical administration include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia, and mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

Compositions suitable for topical administration to the skin may be presented as ointments, creams, gels and pastes comprising one or more compounds of the present invention and a pharmaceutically acceptable carrier. A suitable topical delivery system is a transdermal patch containing the ingredient to be administered.

Compositions suitable for rectal administration may be presented

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as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Compositions suitable for nasal administration wherein the carrier is a solid include a coarse powder having a particle size, for example, in the range 20 to 500 microns which is administered in the manner in which a compound is inhaled, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid, for administration, as for example, a nasal sprain or as nasal drops, include aqueous or oily solutions of the active ingredient.

Compositions suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed

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ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

For example, solid dose forms that can be used for oral administration include capsules, tablets, pills, powders and granules. In such solid dose forms, the active ingredient, i.e., the cassettes with the E2F responsive promoter and nucleic acid segment encoding a heterologous protein is mixed with at least one inert carrier such as sucrose, lactose or starch. Such dose forms can also comprise additional substances other than inert diluents, e.g., lubricating agents, such as magnesium stearate. Furthermore, the dose forms in the case of capsules, tablets and pills may also comprise buffering agents. The tablets, capsules and pills can also contain time-release coatings to release the particles over a predetermined time period.

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For parenteral administration, one typically includes sterile aqueous or non-aqueous solutions, suspensions or emulsions in association with a pharmaceutically acceptable parenteral vehicle. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils such as olive oil and corn oil, gelatin and injectable organic esters, such as ethyl oleate. These dose forms may also contain adjuvants such as preserving, wetting, emulsifying and dispersing agents. They may be sterilized by, for example, filtration through a bacterial-retaining filter, by incorporating sterilizing agents into the composition, by irradiating the compositions, etc., so long as care is taken not to inactivate the active ingredient (e.g. a vector). They can also be manufactured in a medium of sterile water or some other sterile injectable medium before use. Further examples of these vehicles include saline, Ringer's solution, dextrose solution and 5% human serum albumin. Liposomes may also be used as carriers. Additives, such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives, may also be used.

The preferred range of active ingredient in such vehicles is in concentrations of about 1 mg/ml to about 10 mg/ml. More preferably, about 3 mg/ml to about 10 mg/ml.

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The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLES

Tumor Cell Line and Animals.

The rat C6 astrocytoma cell line was maintained in Dulbeccos MEM (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin. Adult female Sprague Dawley rats (150-175g) were purchased from Charles River Laboratories (Wilmington, MA).

Recombinant Adenovirus.

The recombinant adenoviruses Ad.E2F1- β gal and Ad.CMV- β gal were constructed by homologous recombination between pJM17, a plasmid containing the genome of adenovirus type 5 (Ad5) with deletions in the E1 and E3 regions, and a shuttle plasmid that includes Ad5 sequences (map units 0.0 to 1.3 and 9.2 to 17.3) and the *E. coli* lacZ gene driven by either the cytomegalovirus (CMV) early gene enhancer/promoter or the E2F-1 promoter. The E2F-1 promoter utilized

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in Ad.E2F1- β gal contains the upstream region of the E2F-1 gene (basepairs -218 to +51), a segment containing 4 intact E2F (two imperfect palindromes) and 4 Sp1 consensus sequences. Construction of the Δ E2F-1 promoter and the Ad.CMV-tk vector has been previously described 13, 25. The recombinant viruses were plaque purified and propagated in 293 cells as previously described 26. The structure of the resulting recombinant vectors were confirmed by restriction enzyme digestion and sequencing. The virus stocks were purified by two cesium chloride ultracentrifugations, dialyzed against 10% glycerol, 10 mM Tris (pH 8.0) and 1 mM MgCl₂ (viral vehicle) and stored at -80°C. Virus titers were determined as plaque forming units (pfu) assayed in semisolid cultures of 293 cells.

In Vitro Studies

Cell Cycle Inducibility

C6 cells were serum starved (0.5 % fetal bovine serum) for 48 h and then transduced by either Ad.E2F1- β gal, Ad.CMV- β gal or Ad.dE2F1- β gal at a multiplicity of infection (MOI) of 100. Serum starved cells were split into two groups 48 h later, one refed with 10% FBS and the other maintained in low serum. Cells were harvested at the indicated time

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points, fixed with ethanol, stained with propidium iodide (P.I.) or with an anti.- β -galactosidase antibody (Boehringer-Manheim) and analyzed by ELISA.

Cytotoxicity Assays

C6 glioma cells were seeded in 75 cm² tissue culture plates, washed, and infected with viral vectors at various MOIs for 1 h in 0.5% media. Cells were then washed, and cultured in normal media for additional 12 h followed by replating at a density of 500 cells/well in 96-well tissue culture plates containing GCU (Cytovene, Hoffman-La Roche, N.J.) at various concentrations. The cells were fixed 72 h later with the addition of formaldehyde (3.7% final), washed extensively with water, and stained with 200 ml of 0.05% methylene blue for 15 min. Unbound dye was washed away, and bound dye was released for quantitation by addition of 200 Mg/Ml 0.33 N HCl for 15 min. Absorbance at 600 nm (A600) was determined on a microplate reader after mixing for 5 sec.

Animal Studies

Implantation of intracerebral tumors/Vector injections.

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C6 glioma cells were stereotactically implanted into the right caudate nucleus of Sprague Dawley rats using a modification of the method of Kobayashi 27. In brief, rats (150-175 g) were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) and placed in a small animal stereotaxic frame (Kopf Instruments). A sagittal incision was made through the scalp to expose the skull and a small burr hole was made 1.3 mm posterior and 4 mm to the right of the bregma. Twenty thousand tumor cells, suspended in 10 Ml of HBSS, was injected with a 701 Hamilton syringe over 30 seconds to a depth of 4.5 mm. The needle was left in place for one minute and then withdrawn slowly. The hole in the skull was plugged with bone wax and the incision was closed with surgical clips (Ethicon Plus). This method resulted in a 100% yield of intracerebral tumors with relatively little leptomeningeal or intraventricular spread. For the tk studies, Ad.CMV-tk (5×10^8 pfu), Ad.E2F1-tk (5×10^8 pfu), or Ad.E2F1- β gal (5×10^8 pfu) was injected into 6 d old established gliomas followed by intraperitoneal injections of GCV (15 mg/kg) or saline twice a day for 7 d.

Ad.E2F1- β gal (10^9 pfu), Ad.CMV- β gal (10^9 pfu), or Ad.E2F1- β gal (10^9 pfu), was stereotactically injected into normal brain or 14 day old intracerebral C6 gliomas utilizing the same coordinates and methods

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described above. Animals were sacrificed 3 days later and brains were harvested for further studies.

Hepatectomy and Intrahepatic Vector Injections

Sprague Dawley rats (150-175 g) were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). The hepatic artery, portal vein and bile ducts leading to the middle and left lobes of the liver were identified and ligated, allowing removal of these two lobes amounting to a 70% hepatectomy. Forty eight h following hepatectomy or sham operation (controls), Ad.E2F1- β gal (10^9 pfu) or Ad.CMV- β gal (10^9) were injected into the right femoral vein. Animals were sacrificed 4 d later and livers were harvested for further studies.

Tissue Preparation

Animals were sacrificed by pentobarbital overdose followed by cardiac perfusion, first with ice cold PBS, followed by 4% paraformaldehyde/PBS. Brain or liver tissue was harvested whole and further fixed in 4% paraformaldehyde/PBS for a further 2 h at 4°C, and

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then sequentially washed over 3 nights in 10%, 20%, and 30% sucrose in PBS/2 mM MgCl. Brain was then cut coronally along the needle track and liver lobes cut lengthwise and slices used directly for whole mounts or embedded in O.C.T. compound (Tissue Tek) and frozen for thin sections.

For X-gal straining of whole mounts, the fixed samples were rinsed once with cold PBS and incubated in PBS solution containing 2 mM CaCl₂, 0.01% sodium deoxycholate and 0.02% NP-40 for 10 min at 4°C. Samples were then stained for 6 h at 37°C in the same solution containing 5 mM K₃(Fe(CN)₆, 5 mM K₄(Fe(CN)₆ and 1 mg/ml X-gal (5-βromo-4-chloro--5-indolyl-β-D-galatopyranoside), mounted and photographed.

For thin sections, 6 μm sections were cut using an IEC Minotome cryostat and placed on poly-lysine coated slides. Slides were allowed to air dry and stored at -20°C. For staining of liver sections, slides were briefly washed in PBS, fixed with cold acetone and allowed to air dry. X-gal staining was then carried out overnight (18h) with the X-gal staining solution as described above. Anti PCNA primary antibody (PC10, Dako

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Co.) or polyclonal anti-adenovirus anti-serum was diluted 200-fold in PBS/BSA/Tween-20 and applied for 30 min followed by vigorous washing (Chemicon Int. Inc, Temecula, CA.). A biotinylated horse anti-mouse IgG secondary antibody was used for visualization utilizing a peroxidase ABC system (Vectastain, Vector Laboratories) and DAB with nickel enhancement as the substrate as previously described. Stained sections were counterstained with nuclear fast red (Vector Laboratories) to highlight cell nuclei. The anti-adenovirus monoclonal was detected using a goat-antimouse IgG-FITC-conjugated antibody (Chemicon Int. Inc, Temecula, CA.), and visualized under fluorescent microscopy. Standard hematoxylin and eosin (H+E) staining of brain sections was also carried out after fixing the slides in cold methanol.

E2F-1 is a ubiquitously expressed, growth regulated, gene which exhibits peak transcriptional activity in S-phase^{11, 12}. The E2F-1 promoter contains 4 E2F binding sites which act as repressor elements in quiescent cells^{5, 13-16}. We constructed an adenoviral vector that utilizes the E2F-1 promotor (basepair -218 to +51; a segment containing 4 intact E2F consensus sequences) to drive expression of an E coli β -galactosidase gene, placed in the E1 region of an E1 /E3 deleted adenoviral vector (Fig. 1) as previously described^{17, 18}. Transduction of

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proliferating C6 glioma cells by Ad.E2F1- β gal resulted in high level expression of β -galactosidase activity in a titer-dependent manner (data not shown). We next examined whether Ad.E2F1- β gal could mediate cell cycle-dependent transgene expression in vitro. C6 cells were serum starved, transduced by either Ad.E2F1- β gal or Ad.CMV- β gal (an identical vector except for the presence of a constitutively active cytomegalovirus early promotor rather than the E2F-1 promotor; Fig. 1), and then re-exposed to serum. As demonstrated in Fig. 2A and B, serum starvation resulted in a relatively constant number of cells in S phase (~10%), without significant cytotoxicity, whereas re-exposure to serum resulted in an increased S phase fraction to 25%, 46%, and 60% at 16, 20, and 28 h, respectively. β -galactosidase activity was approximately 20 fold above background in the Ad.CMV- β gal transduced, serum starved cells, and remained constant at this level following re-exposure to serum. In contrast, Ad.E2F1- β gal transduced, serum starved cells expressed relatively low levels of β -galactosidase activity. When these cells were re-exposed to serum, however, β -galactosidase activity was rapidly induced 8, 14, and 20 fold over baseline at 16, 20, and 28 hours, respectively (Fig. 2B; $p < 0.001$). These data are consistent with the suggestion that Ad.E2F1- β gal mediated transgene expression is induced following entry into and/or progression through the cell cycle. The fact that the level of β -galactosidase expression in Ad.E2F1- β gal transduced cells 24 h

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following serum re-feeding was similar at equivalent MOIs to that seen in cells transduced by Ad.CMV- β gal, suggests that the induced/de-repressed E2F-1 promotor can mediate high level transgene expression. Similar results were obtained with a panel of human malignant glioma cell lines, suggesting that the observations made with the C6 cells were not cell line specific (data not shown).

Malignant gliomas represent an excellent model for therapeutic gene transfer given the ability to inject viral vectors directly into these relatively localized tumors¹⁹⁻²¹. To test the tumor selective properties of the Ad.E2F1- β gal vector in vivo, we stereotactically injected Ad.E2F1- β gal or the control Ad.CMV- β gal into normal rat brains or brains with established C6 gliomas, and sacrificed the animals 3 d later. As can be seen in Fig. 3A, injection of Ad.CMV- β gal into normal brain resulted in high levels of β -galactosidase activity in brain adjacent to the needle tract, and along ependymal surfaces. Injection of Ad.CMV- β gal into a large established C6 glioma resulted in widely dispersed β -galactosidase activity predominantly in the tumor and in edematous brain infiltrated by tumor, but also in normal adjacent brain and along ependymal surfaces (Fig. 3B) as previously described²². In marked contrast, normal rat brains injected with Ad.E2F1- β gal resulted in virtually no β -galactosidase staining, whereas injection into an established C6 glioma

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resulted in extensive staining of the tumor itself, with little or no staining in the adjacent normal brain (Fig. 3C, 3D, respectively).

Two, non-mutually exclusive, possibilities could be invoked to account for the enhanced β -galactosidase activity in tumor tissue relative to normal tissue following injection with Ad.E2F1- β gal. One explanation, given the cell cycle data described above, would be that a higher fraction of glioblastoma cells are cycling than are normal cells found in the surrounding, mitotically quiescent brain. A second explanation might be that glioma cells, by virtue of pRB inactivation, contain high levels of 'free' E2F and lack pRB/E2F transcriptional repressor complexes, in which case even mitotically active normal cells might not achieve the high level of transgene expression observed in tumor cells. To address these possibilities, we performed two different sets of experiments. In the first set of experiments, rats underwent partial hepatectomy followed by injection of either Ad.CMV- β gal (10^9 pfu) (Fig. 4 A,C,E) or Ad.E2F1- β gal (10^9 pfu) (Fig. 4B, D, F) into the femoral vein 48 h later. Four days later, livers were harvested and stained for β -galactosidase activity, proliferating nuclear antigen (PCNA), and for the adenovirus fiber protein (Fig. 4). Approximately 30% of the hepatocytes were positive for PCNA under these conditions, confirming the presence of a large number of normal proliferating cells in the regenerating livers

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(Fig. 4C,D). Ad.CMV- β gal transduced livers demonstrated diffuse β -galactosidase staining with no apparent difference between PCNA positive versus negative cells (Fig. 4A). In contrast, Ad.E2F1- β gal transduced hepatocytes, including those which were PCNA positive, did not express detectable levels of β -galactosidase (Fig. 4B). Detection of adenovirus fiber protein in the liver sections demonstrated similar levels of hepatic transduction by both the Ad.CMV- β gal and Ad.E2F1- β gal vectors (Fig. 4E,F). Similarly, Ad.CMV- β gal, but not Ad.E2F1- β gal, produced high levels of β -galactosidase activity following transduction of non-hepatectomized livers (data not shown). These data suggest that the high level E2F-1 promotor-mediated transgene expression in vivo is not merely a function of active cell cycling.

In the second set of experiments, we constructed a virus identical to Ad.E2F1- β gal, except that mutations were introduced into the two E2F-binding site within the E2F-1 promotor (Ad. Δ E2F1- β gal). These mutations have previously been shown to render the mutated E2F-1 promotor unresponsive to E2F in vitro¹³ In experiments similar to those outlined in Fig. 2, transduction of serum starved and refed C6 cells in vitro by Ad. Δ E2F1- β gal resulted in constitutive transgene expression independent of cell cycle (data not shown). When Ad. Δ E2F1- β gal was stereotactically injected into non-tumor-bearing rat brain, β -

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galactosidase activity was readily apparent in normal cells (Fig. 3E). This is in marked contrast to the lack of β -galactosidase expression in normal cells when the vector contained the intact E2F-consensus sequence (Ad.E2F1- β gal; Fig. 3C). Interestingly, Ad. Δ E2F1- β galmediated β -galactosidase expression in established gliomas in vivo was less than that seen with Ad.E2F1- β -gal Fig. 3F). These data suggest that the E2F-responsive sequences within the E2F-1 promoter are essential for the tumor selective properties of the vector. Furthermore, they strongly suggest that the tumor selectivity of the E2F responsive vector is due to a combination of factors, namely, repression in normal tissue by pRB/E2F complexes and activation in tumor tissue due to loss of pRB repressor complexes and an excess of free E2F.

To confirm that the tumor-selective properties of this vector could be successfully utilized for expression of a therapeutic gene, we constructed vectors containing the herpes thymidine kinase (tk) gene driven by either the E2F-1 promotor (Ad.E2F1-tk) or the CMV promotor (Ad.CMV-tk). In vitro transduction of C6 cells by Ad.CMV-tk or Ad.E2F1-tk, demonstrated that both vectors equally sensitized glioma cells to ganciclovir (GCV) ($LD_{50} = 0.05$ MM; Fig. 5A). In order to confirm the activity of the vector in vivo, Ad.CMV-tk or Ad.E2F1-tk were stereotactically injected into 7 day old intracerebral C6 gliomas, followed

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by systemic GCV treatment for 1 week (Fig. 5B). There was no significant difference in survival in animals treated with either Ad.CMV-tk or Ad.E2F1-tk plus GCV (> 60% of animals without evidence of tumor > 3 months from treatment), although animals in both these groups lived significantly longer than animals from the control groups (Log rank analysis of Kaplan-Meier survival curves, $p < 0.0001$; Fig. 5B).

Since the evaluation of normal brain toxicity in tumor-bearing animals can be difficult as a result of changes induced by necrotic tumor tissue, we elected to evaluate vector-mediated normal tissue toxicity in the brains of non-tumor bearing rats. One week following the injection of either Ad.CMV-tk or Ad.E2F1-tk and systemic GCV therapy, non-tumor bearing brains were harvested and pathologically evaluated. Consistent with reports in nonhuman primates, stereotactic injection of a tk constitutively expressing adenoviral vector (Ad.CMV-tk) plus GCV resulted in extensive areas of local brain necrosis, inflammation and hemorrhage (Fig. 5C)²³. In marked contrast, treatment with Ad.E2F1-tk and GCV resulted in no obvious normal tissue toxicity, except that from the local trauma of the stereotactic injection (indistinguishable from sham injected animals). These studies confirm that an E2F-responsive adenoviral vector can mediate tumor selective cytotoxic transgene expression with similar anti-tumor efficacy, but with significant less

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normal tissue toxicity, than that of constitutively expressing vectors.

These results indicate that the activity of E2F responsive promoters in tumor cells exceeds that achieved in mitotically active normal cells, and this difference can be exploited to target tumor-selective gene expression in vivo. This may be particularly relevant for glioblastomas where the p16-cdk4/cyclin D1-Rb signal pathway is deregulated in the majority of cases^{2, 9, 10, 24}.

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All the references mentioned herein are incorporated by reference.

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We claim:

1. A nucleic acid cassette comprising:
 - (1) an E2F responsive promoter, wherein said promoter in the presence of "free" E2F, expresses a gene operably linked to said promoter;
 - (2) a nucleic acid segment containing a nucleic acid sequence of interest operably linked to said E2F responsive promoter, wherein said gene of interest is a positive potentiator or a negative potentiator .
2. The nucleic acid cassette of claim 1, wherein the nucleic acid sequence of interest encodes a negative potentiator selected from the group consisting of an antibody, a suicide protein, a dominant negative mutant, and a cytotoxic agent.
3. The nucleic acid cassette of claim 1, wherein the nucleic acid segment is encodes a cytotoxic protein or cytotoxic fragment thereof.
4. The nucleic acid cassette of claim 3, wherein the nucleic acid segment encodes at least Domain III of *Pseudomonas* exotoxin A.
5. The nucleic acid cassette of claim 1, wherein the E2F responsive promoter is selected from the group of promoters consisting of E2F1 promoter, dihydrofolate reductase promoter, DNA polymerase α promoter, c-myc

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promoter and B-myb promoter.

6. The nucleic acid cassette of claim 5, wherein the promoter is the human wild type E2F-1 promoter.
7. A cassette containing the nucleic acid cassette of claim 1.
8. The vector of claim 7, wherein the vector is selected from the group consisting of chemical conjugates, fusion proteins containing a targeting moiety and nucleic acid binding moiety, retroviral vectors and DNA viral vectors.
9. The vector of claim 8, wherein the vector is a DNA viral vector selected from the group consisting of herpes viral vectors, adenoviral vectors and adeno-associated viral vectors.
10. The vector of claim 8, wherein the vector is an adenoviral vector.
11. The vector of claim 10, wherein the nucleic acid sequences of interest encodes a negative potentiator.
12. The vector of claim 11, wherein the negative potentiator is a suicide protein or a cytotoxin.
13. The vector of claim 12, wherein the negative potentiator is a suicide protein and the suicide protein is HSV thymidine kinase.

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14. A method of selectively targetting a malignant cell which comprises adding an effective amount of the nucleic acid cassette of claim 1 to a medium containing the malignant cell under conditions where the nucleic acid cassette can transduce the cell and waiting until the nucleic acid cassette transduces the malignant cell.

15. The method of claim 14, wherein the nucleic acid cassettes is present in a viral vector or nucleic acid delivery system.

16. The method of claim 14, wherein the malignant cell is a solid tumor.

17. The method of claim 11, wherein the solid tumor is a glioma.

18. The method of claim 17, wherein the nucleic acid cassettes is present in a vector, wherein the vector is an adenovirus vector or a herpes virus vector.

19. The method of claim 16, wherein the nucleic acid sequence of interest encodes a negative potentiator.

20. The method of claim 19, wherein the negative potentiator is a suicide gene or a cytotoxin.

21. The method of claim 20, wherein the negative potentiator is a suicide gene.

22. The method of claim 21, wherein the suicide gene is HSV thymidine kinase.

23. The method of claim 22, wherein the negative potentiator is

a cytotoxin.

24. The method of claim 23, wherein the cytotoxic contains at least Domain III of *Pseudomonas* exotoxin A.

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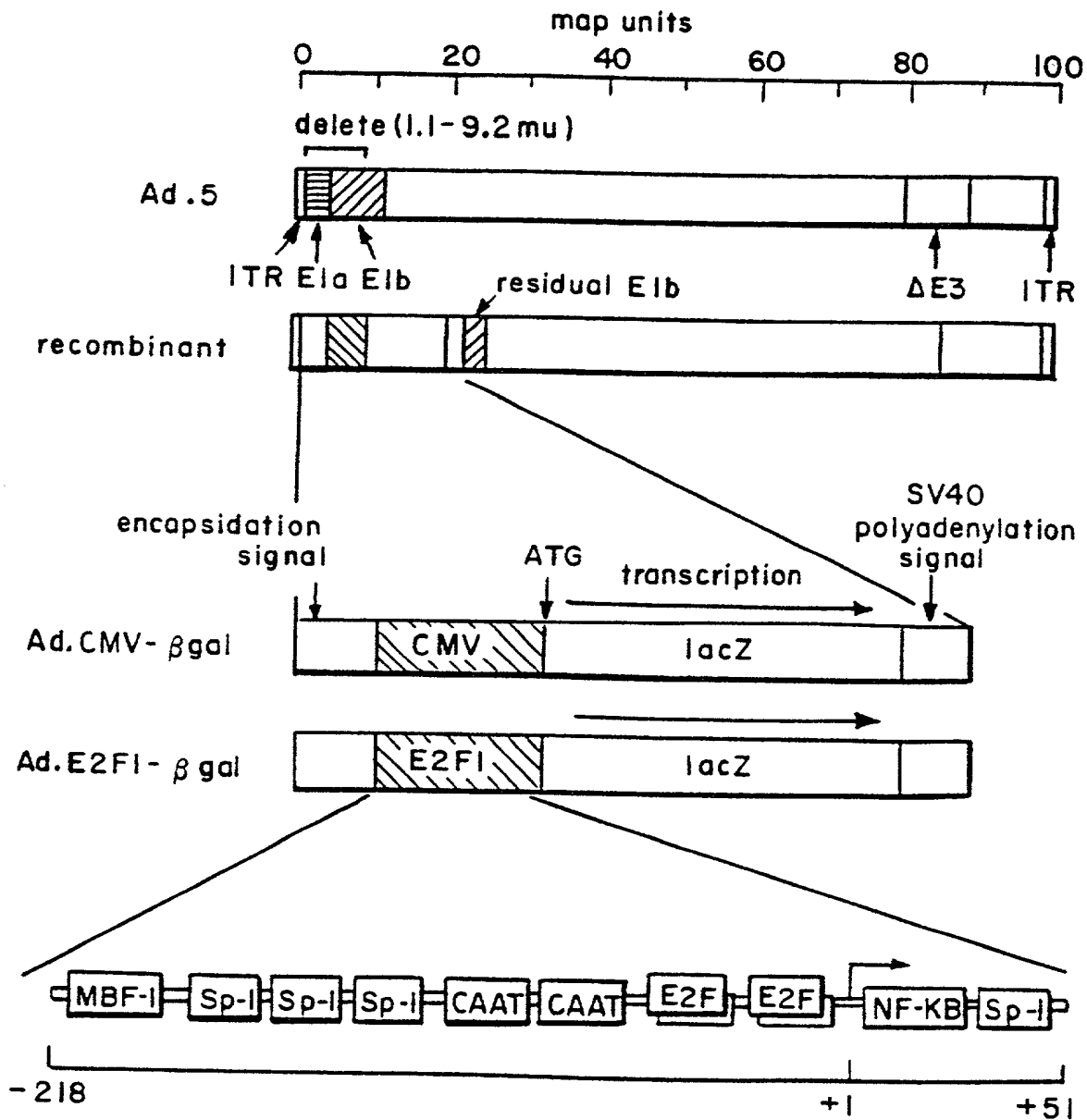


FIG. 1

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FIG.2A

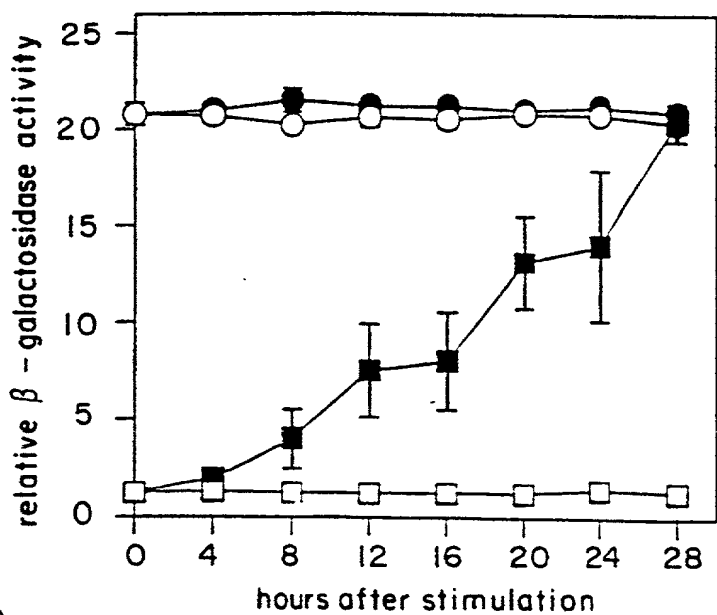
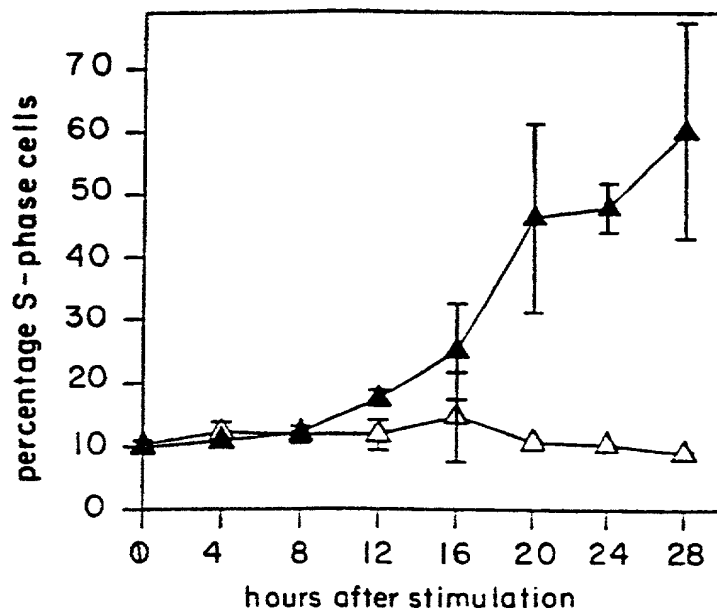


FIG.2B

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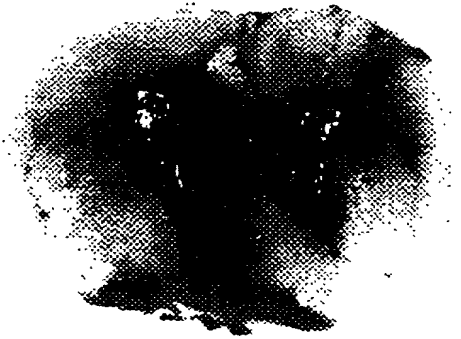


FIG. 3A



FIG. 3B

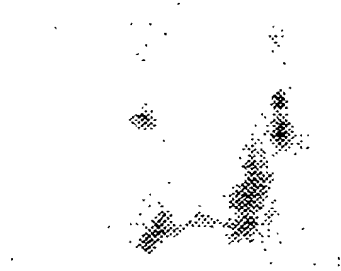


FIG. 3C



FIG. 3D



FIG. 3E

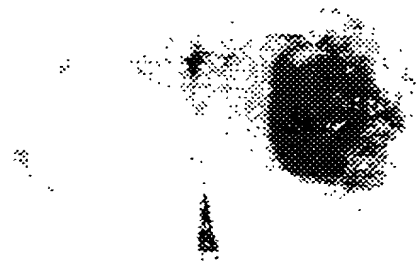


FIG. 3F

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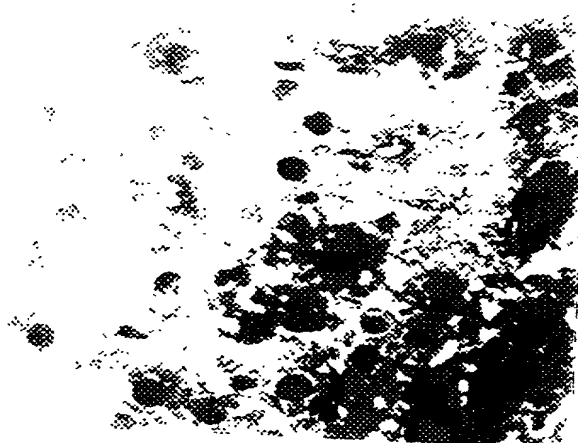
FIG. 4A



FIG. 4B



FIG. 4C



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FIG.4D

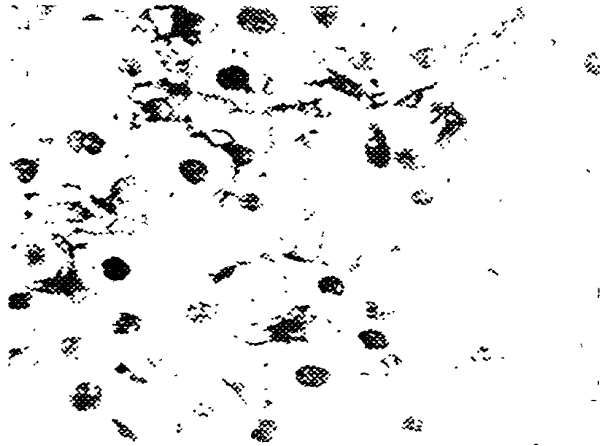


FIG.4E

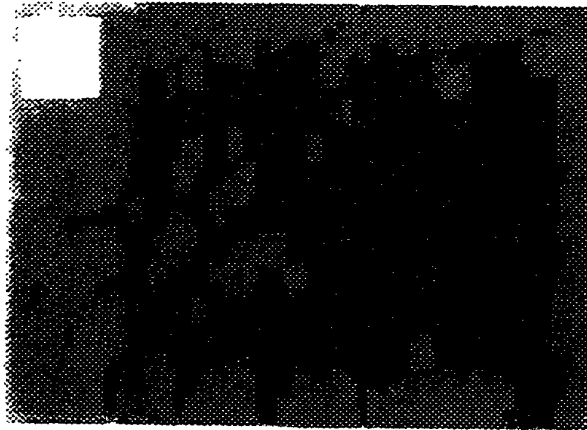


FIG.4F



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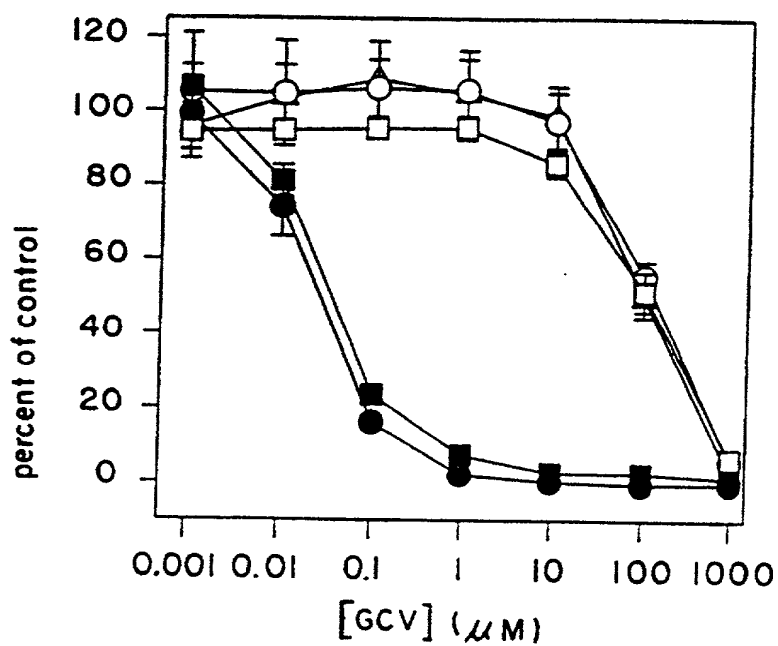


FIG. 5A

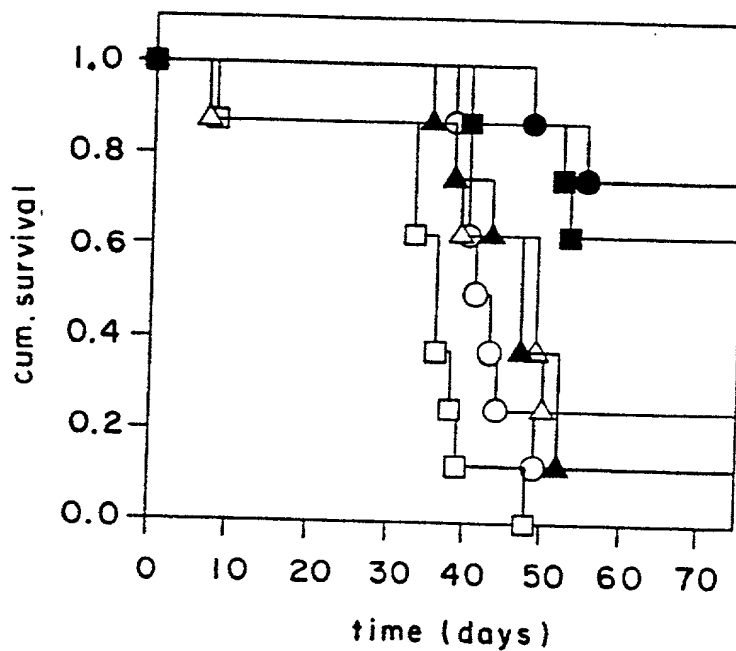


FIG. 5B

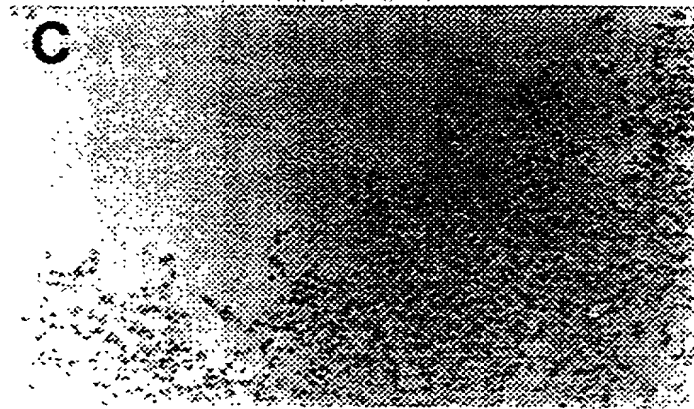


FIG. 5C

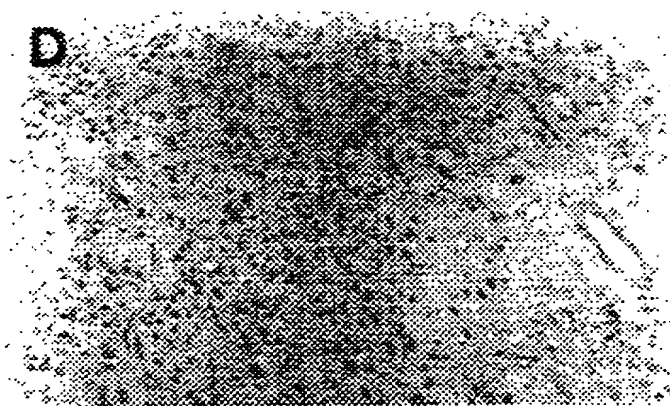


FIG. 5D

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed at 201) below or an original, first and joint inventor (if plural names are listed at 201-208 below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD OF TARGETING MALIGNANT CELLS USING AN E2F RESPONSE PROMOTER

which is described and claimed in:

- ☐ the specification attached hereto.
- ☐ the specification in U.S. Application Serial Number _____
filed on _____.
- X the specification in PCT international application Number PCT/US97/17143, filed on
24 September 1997; and was amended on _____.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign/PCT Applications and Any Priority Claims Under 35 U.S.C. §119:			
Application No.	Filing Date	Country	Priority Claimed Under 35 U.S.C. §119?
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Prior U.S. Applications or PCT International Applications Designating the U.S-Benefit

Under 35 U.S.C. §120

U.S. Applications		Status (Check One)		
Application Serial No.	U.S. Filing Date	Patented	Pending	Abandoned
PCT Applications Designating the U.S.				
Application No.	Filing Date	U.S. Serial No. Assigned		
PCT/US97/17143	24 September 1997			X

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. §119(e))

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Applicant	Provisional Application Number	Filing Date
W. Kaelin, Jr. et al.	60/026,959	24 September 1996

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Ronald I. Eisenstein

(Reg. No. 30,628)

David S. Resnick

(Reg. No. 34,235)

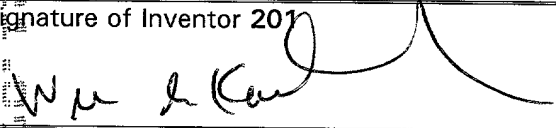
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SEND CORRESPONDENCE TO: Ronald I. Eisenstein Nixon Peabody LLP 101 Federal Street Boston, Massachusetts 02110	DIRECT TELEPHONE CALLS TO: Ronald I. Eisenstein (617) 345-6054
--	---

2 0 1	FULL NAME OF INVENTOR	LAST NAME Kaelin, Jr.	FIRST NAME William	MIDDLE NAME
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	POST OFFICE ADDRESS	POST OFFICE ADDRESS 12 Commonwealth Avenue	CITY Boston	STATE OR COUNTRY AND ZIP CODE Massachusetts 02116

2 0 2	FULL NAME OF INVENTOR	LAST NAME FINE	FIRST NAME Howard	MIDDLE NAME A.
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	POST OFFICE ADDRESS	POST OFFICE ADDRESS 352 Commonwealth Avenue, Unit 4	CITY Boston	STATE OR COUNTRY AND ZIP CODE Massachusetts 02115

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201 	Date: 8/12/99
Signature of Inventor 202	Date:

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed at 201) below or an original, first and joint inventor (if plural names are listed at 201-208 below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

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Prior Foreign/PCT Applications and Any Priority Claims Under 35 U.S.C. §119:			
Application No.	Filing Date	Country	Priority Claimed Under 35 U.S.C. §119?
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Prior U.S. Applications or PCT International Applications Designating the U.S-Benefit Under 35 U.S.C. §120					
U.S. Applications			Status (Check One)		
Application Serial No.	U.S. Filing Date	Patented	Pending	Abandoned	
PCT Applications Designating the U.S.					
Application No.	Filing Date	U.S. Serial No. Assigned			
PCT/US97/17143	24 September 1997			X	

**CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. §119(e))**

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(Reg. No. 30,628)

David S. Resnick

(Reg. No. 34,235)

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--	---

2 0 1	FULL NAME OF INVENTOR	LAST NAME Kaelin, Jr.	FIRST NAME William	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY Boston	STATE OR FOREIGN COUNTRY Massachusetts	COUNTRY OF CITIZENSHIP USA
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 12 Commonwealth Avenue	CITY Boston	STATE OR COUNTRY AND ZIP CODE Massachusetts 02116

2-00

2 0 2	FULL NAME OF INVENTOR	LAST NAME FINE	FIRST NAME Howard	MIDDLE NAME A.
	RESIDENCE & CITIZENSHIP	CITY Boston	STATE OR FOREIGN COUNTRY Massachusetts MA	COUNTRY OF CITIZENSHIP USA
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 352 Commonwealth Avenue, Unit 4	CITY Boston	STATE OR COUNTRY AND ZIP CODE Massachusetts 02115

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Signature of Inventor 201	Date:
Signature of Inventor 202 	Date: 9/10/99